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From words to literature in structural proteomics

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Technical advances on several frontiers have expanded the applicability of existing methods in structural biology and helped close the resolution gaps between them. As a result, we are now poised to integrate structural information gathered at multiple levels of the biological hierarchy — from atoms to cells — into a common framework. The goal is a comprehensive description of the multitude of interactions between molecular entities, which in turn is a prerequisite for the discovery of general structural principles that underlie all cellular processes.

The structures of individual macromolecules are often uninformative about function if taken out of context. Just as words must be assembled into sentences, paragraphs, chapters and books to make sense, vital cellular functions are performed by structured ensembles of proteins (that is, complexes), not by freely diffusing and occasionally colliding proteins^{1,2}. Frequently, these complexes comprise ten or more subunits (Fig. 1). Recent proteomics studies with yeast, for example, have indicated that the number of complexes that exist at least transiently in a cell has been underestimated. The techniques of isolation and purification that are traditionally used in biochemistry tend to select for the most robust complexes, whereas the more weakly interacting and transient complexes escape attention and, therefore, analysis.



Figure 1 Illustration of the size range of biomolecular structures solved by X-ray crystallography and the size distribution of structures contained in the Protein Quaternary Structure (PQS) database (<http://pqs.ebi.ac.uk>). [Full legend](#)

[High resolution image and legend \(54k\)](#)

In recent years, two trends have emerged in structural biology: efforts to achieve a comprehensive coverage of individual protein structures (so-called structural genomics) and efforts to analyse the structures of large complexes^{2,3}. Structural biology has flourished in the wake of technological innovations in fields as diverse as biochemistry, molecular biology, computational biology, computer hardware and software, nuclear magnetic resonance (NMR) magnets and optimized pulse sequences, and synchrotron

radiation, as well as advances in light and electron microscopy (EM) instrumentation and in detector technology. Notwithstanding the value and importance of the individual techniques, a combination of approaches is likely to be more powerful than any single method alone. In this review we discuss some integrated strategies and tactics that can be used for characterizing molecular complexes and for describing their interactions in a cellular context.

The challenge of myriads of complexes

Given the average length of 466 residues for a yeast protein and 173 residues for a domain in the CATH database⁴ (a hierarchical classification of protein domain structures), one can estimate that, on average, a protein is folded into approximately two domains. In the evolution of proteins, domains are important units that are shuffled, duplicated, and fused into larger proteins. Although the universe of distinct amino acid sequences is essentially unlimited, the number of different folding patterns for the domains is not. Extrapolation based on the existing databases of protein sequence and structure indicates that most of the natural domain sequences assume one of a few thousand folds⁵, of which ~1,000 are already known⁴.

In contrast to the folds, there are no satisfactory estimates of the number of different non-covalent macromolecular complexes with a unique structure and biological function. Such estimates are non-trivial to make because of the multitude of the component types (for example, proteins and nucleic acids), and the varying lifespan of the complexes (for example, transient complexes such as those involved in signalling, and stable complexes such as the ribosome). In addition, there is no self-evident definition of what is a 'complex' and whether two complexes are of different types. In an extreme view, a whole cell or even an organism may be seen as a single giant complex.

The Protein Quaternary Structure (PQS) database currently contains ~10,000 structurally defined protein assemblies of presumed biological significance, derived from a variety of organisms (<http://pqqs.ebi.ac.uk/pqqs-doc.shtml>); each assembly consists of at least two protein chains. Just like the folds, these assemblies can be organized into ~3,000 groups such that the members of the same assembly group share more than 30% sequence identity between the equivalent constituent protein chains (Fig. 1).

The most comprehensive information about both stable and transient protein complexes exists for the yeast proteome of ~6,200 proteins. But even for this model genome, uncertainties in the number, types and sizes of the complexes arise because of the difficulty in unravelling physical interactions from functional links⁶, binary from multiple physical interactions, transient from stable interactions, and direct interactions from indirect physical interactions through intermediates. In addition, each method may be impacted differently by the localization of the proteins in the cellular environment and may have significantly different rates of false positives and negatives.

The Munich Information Center for Protein Sequences (MIPS)⁷ and Yeast Proteome Database (YPD)⁸ list ~11,000 binary interactions and functional links documented by focused, small-scale experiments⁹, corresponding on average to ~3.5 partners per protein. Large-scale yeast two-hybrid data^{10, 11} indicate 1.7 partners per protein, when artefactual interactions are removed from consideration¹². On the other hand, the affinity purification of 1,739 yeast protein baits indicated 232 distinct complexes of an average size of 7.5 proteins, suggesting that the whole yeast proteome may contain ~900 complexes¹³. A comparison of these purified complexes against the complexes of known structure revealed that most of them are stable as opposed to transient, whereas the reverse applies to the interactions detected by the yeast two-hybrid methods¹⁴⁻¹⁷. Only one-third of the binary interactions and functional links obtained by more than one high-throughput method occur in the curated MIPS/YPD set of the ~11,000 binary interactions and links, suggesting that the lower bound on the binary protein-protein interactions and functional links in yeast is ~30,000 (refs 9, 18). This number corresponds to ~9 protein partners per protein or 3.6 protein partners per domain, not necessarily all direct or at the same time.

The human proteome may have an order of magnitude more complexes than the yeast cell; and the number of different complexes

across all relevant genomes may be several times larger still. Therefore, there may be thousands of biologically relevant macromolecular complexes whose structures are yet to be characterized¹⁹.

Towards an unabridged dictionary of proteins

Currently, X-ray crystallography is the most prolific technique for the structural analysis of proteins and protein complexes, and it still is the 'gold standard' in terms of accuracy. While this technique has provided the majority of structures in the database of biomolecular structures, the fraction determined by NMR spectroscopy is also significant (currently 14%)²⁰. From the earliest structures of myoglobin and haemoglobin through the recent studies of RNA polymerase²¹, the ribosomal subunits²²⁻²⁴, and the complete ribosome and its functional complexes²⁵, these structural data have contributed tremendously to our understanding of biology at the molecular level. As seen in [Fig. 1](#), the sizes of the structures determined by X-ray crystallography range from small proteins, such as the 100-residue PDZ domain, which recognizes and binds other proteins, to the 70S ribosome, which consists of 52 proteins and 3 RNA molecules, and has a relative molecular mass of $\sim 2,500,000$ (M_r 2,500K).

Crystallography requires that milligram quantities of a pure and monodisperse protein can be prepared, and that the protein can be induced to form three-dimensional (3D) periodic arrays (that is, crystals). Therefore, almost all proteins used for structural studies are expressed in heterologous expression systems. Bacterial expression systems are simple and rapid, in addition to being amenable to incorporation of selenium as an anomalous scatterer for determining phases. However, overexpression in bacteria may not produce large amounts of the correctly folded protein, or the protein may lack appropriate post-translational modification. To overcome such limitations, there are a number of strategies that involve using genes from different species, altering constructs, screening for solubility, and utilizing different cellular or cell-free expression systems. The constructs can be altered in numerous ways, such as by the addition of tags, separation of proteins into domains, or the use of gene shuffling methods.

Once the proteins are expressed and purified, it is necessary to form crystals of sufficient quality to collect high-resolution (at least 2.5 Å) data for structure determination. Because crystallization conditions cannot be pre-determined, it is necessary to screen a wide range of conditions (such as pH, salt, protein concentration and co-factors). Over the past few years, this area has benefited enormously from automation and technologies allowing the use of small sample volumes²⁶. Particularly for proteins and protein complexes with low yields, the ability to screen more conditions at the required protein concentration is critical.

Currently, most biological crystallography experiments are done at synchrotrons, where the brightness (high flux of well-collimated X-rays) and tunability expand the capabilities and throughput enormously. The increase in the amount and diversity of structural data that have been obtained in the past five to ten years has been greatly enhanced by the availability of beamlines and detectors of increasing performance. As the systems have evolved from primitive to 'user-friendly', robotic crystal mounting and alignment systems have also been implemented at beamlines²⁷ to increase the throughput and productivity of these expensive and oversubscribed resources. Once data are obtained, usually in one to several hours on modern third-generation synchrotrons, the analysis of the primary data can also be completed in several hours.

Increasingly, therefore, structures are solved within hours after data collection begins, although most structures still need a great deal more time for the screening of crystals, full data collection, and the processing and analysis that leads to an accurate high-resolution structure. Nevertheless, as the beamlines become more automated and as higher-level control and processing software is further developed, it is becoming feasible to integrate the data collection, processing and analysis steps — from crystal mounting through structure refinement — to form a 'pipeline' of information for structure determination. The technological advances, such as third-generation synchrotrons and charge-coupled device (CCD)-based detectors, have also been critical for the success of structure determinations of several large complexes and viruses. Crystals from such samples typically have very large unit-cell dimensions and diffract even more weakly than 'ordinary' biomolecular crystals.

Recently, several international efforts have been initiated to determine the structures of at least one member from each domain family, such that the structures of the remaining protein sequences can be characterized based on their similarity to the known structures^{28, 29}. Structural genomics aims to construct a taxonomy of protein structures that will serve as a 'dictionary' for the interpretation of the genomic data. In the United States, the Protein Structure Initiative of the National Institute of General Medical Sciences (NIGMS) has funded nine pilot centres to develop high-throughput pipelines for structure determination³⁰. The NIGMS initiative is paralleled by similar efforts in Europe and Japan. Following the success of the genome sequencing programmes, where the use of automation has been important in the increase of productivity, these structural genomics programmes are currently implementing automation of protein production, crystallization, data collection and analysis.

Although it is legitimate to ask how successful structural genomics will be in terms of structures solved versus targets chosen for cloning, a fair assessment at this point in time is difficult. In the early years, it is first necessary to establish the appropriate infrastructure, and it will take time until this investment pays dividends. Success also depends on the choice of targets; there are easy proteins and families, as well as more difficult ones, such as membrane proteins. Whereas success rates of 1–10% per attempted protein are often quoted, this estimate may be misleadingly pessimistic. Many target families have >10 members, a large number of which are usually attempted in parallel. Therefore, the likelihood that at least one of the targeted family members yields a structure is higher than 10%. Whatever the timeframe may be, there is no doubt that structural genomics will make a major contribution to the proteomics dictionary of words and phrases. But words or even phrases alone do not make literature.

Using EM images to produce three-dimensional structures

A powerful advantage of EM is the fact that it is possible to treat images of single molecules in the same way as crystalline arrays. The ability to use non-crystalline particles means, in turn, that it is possible to work with very small quantities of material, the purity need not be at the standard required for crystallization, and specimen tilting (a bottleneck discussed below) is not needed to collect data for a 3D reconstruction. The electron microscope produces images that represent only 2D projections of the specimen, in which all information about the third dimension of the object has been lost. Nevertheless, the full 3D structure of the object can be reconstructed again if one is able to start with many such projections, each showing the object from a different angle³¹. As a result, the unique contributions that can be made by EM include studies of large, complex assemblies without any requirement for crystallization, and, as will be discussed later, even their visualization within whole cells by electron tomography.

Unfortunately, the electrons in a microscope also represent a beam of ionizing radiation that damages the sample while the image is being formed. As a result, it is necessary to limit the electron exposure to a value that is so low that the images have extremely high levels of 'shot noise' (statistical variation in the number of electrons recorded at each point in the image). Equivalent images of separate molecules must therefore be averaged to reduce the statistical noise that is present in each such image.

If the specimen is one molecule thick with all molecules in the same orientation (as in a 2D crystal), the necessary spatial averaging of images is easy. In fact, 3D reconstructions that have been obtained at a high enough resolution to trace the polypeptide chain have all been produced with the use of 2D crystals^{32–38}. Although only ~100 images of highly tilted crystals are needed to produce such a reconstruction, collection of this amount of experimental data is nevertheless slow because the yield of good images drops to 1% or less of that obtained with untilted specimens. As a result, structural studies with 2D crystals have only seldom been taken to a high enough resolution to allow building an atomic model directly into the 3D reconstruction.

Other specimens may exist in the form of long helices or other particles with very high symmetry (for example, icosahedra). These high-symmetry particles usually do not need to be tilted, as the individual particles are naturally rotated by a random amount relative to one another. The number of protein monomers within one such particle remains relatively small, and thus data from many equivalent particles may still have to be averaged to obtain a reconstruction. In practice, such reconstructions have rarely extended beyond about 7–8-Å resolution^{39, 40}. Even so, the ability to visualize elements of secondary structure at this resolution makes it easy to fit a previously determined atomic model of protein monomers into the density. The recent docking of the atomic structure of

tubulin into the EM density map of a complete microtubule⁴¹ illustrates just how precise this docking can be. This type of docking can then provide accurate images of the protein–protein contacts that lead to the assembly of larger macromolecular machines (Fig. 2).



Figure 2 Docking the atomic model of tubulin into the cryo-EM density map of the assembled microtubule. [Full legend](#)

[High resolution image and legend \(68k\)](#)

Because the electron microscope produces images, and not only diffraction intensities, it is possible to determine the positions and relative orientations of randomly distributed, asymmetric macromolecules. The individual images must then be sorted into a large number of distinct classes of views before they can be averaged. This step in the process is illustrated in Fig. 3a, which shows a gallery of 12 different class averages obtained from ice-embedded specimens of *Drosophila melanogaster* tripeptidyl peptidase II (TPP II)⁴². Once a large set of views is in hand, the 3D reconstruction is computed in much the same way as if the average projections had been computed from images of tilted, 2D crystals. As in the example of TPP II that is shown in Fig. 3b, the resulting 3D reconstruction immediately shows how a large, multi-protein complex is assembled from its individual parts. These single particles must be large in size, however, to provide sufficient signal for the alignment at high resolution⁴³. In addition, structure determination by single-particle cryo-EM involves far greater amounts of computation than does structure determination based on 2D crystals or particles with very high internal symmetry (Box 1).



Figure 3 Representative example that illustrates the type of 3D reconstructions that can be obtained with large macromolecular complexes by single-particle cryo-EM. [Full legend](#)

[High resolution image and legend \(29k\)](#)

Although the capabilities of single-particle cryo-EM are powerful, the method still remains slow compared to other structure-determination technologies, such as X-ray crystallography or NMR spectroscopy. Completion of a structure at the modest resolution of ~2 nm currently may require a month or more for data collection and perhaps another month for data processing. If the goal is to obtain a density map in which features of secondary structure are clearly visible, data collection may extend over several months. A further drawback of cryo-EM is the fact that data collection remains a specialist craft that requires many months, even years of training, before one is able to take full advantage of the high performance of modern electron microscopes.

But it is not necessary for data collection to take as long as it currently does, or to be so dependent on the scientist having a high level of acquired technical expertise. Instead, recording a large number of particle images that are invisible to the human eye on the viewing screen involves blindly following a prescribed sequence of repetitive operations. In principle, such a task is better suited for a computer than a human operator. Indeed, automated implementations of single-particle data-collection operations have recently been published^{44, 45}. The next frontier where work has already begun includes automation of the steps in which images of individual particles are selected within digitized micrographs and the data are merged into a 3D reconstruction of the particle. In one recent demonstration, for example, data were collected and a 3D reconstruction was obtained for the tobacco mosaic virus particle at a resolution of ~1 nm in a period of less than 24 hours⁴⁶. Further development of automated data collection and analysis promises to

reduce the turnaround time for producing 3D density maps of large, macromolecular particles from months or years to days or weeks.

The 3D reconstructions obtained by cryo-EM are likely to be used primarily for docking (that is, assembling) atomic-resolution models of component macromolecules into the 3D densities of intact complexes. When the resolution of the density map is high enough to see helices and regions of β -sheet, the docking can be done precisely and with little ambiguity. At lower resolution, however, the docking must be performed with caution, and researchers continue to develop quantitative criteria that can guide the operation⁴⁷⁻⁵⁰. It is therefore fortunate that the throughput of cryo-EM should soon become well matched to the combined throughput of X-ray crystallography and NMR spectroscopy, which are the primary sources of the structures of the individual components. In turn, atomic models of the various assembled components can then be used to interpret each of the recognizable densities that are visualized within whole-cell tomograms.

Hybrid approaches to structure determination

X-ray crystallography may provide high-resolution structures of large complexes, if they can be purified in sufficient quantities and crystallized. Single-particle EM can provide medium-resolution structures (~ 1 nm) of complexes even if only small amounts of material are available and can tolerate some sample heterogeneity. Even so, these 'direct' methods are surely not capable of characterizing the myriads of stable complexes that exist in a cell. In addition, most of the transient complexes cannot be addressed at all with these approaches. Therefore, there is a great need for hybrid methods where both high throughput and highest possible resolution are achieved by integrating information from different sources. This integration should be performed in an objective manner, such that it is reproducible by any expert.

The hybrid assembly of a complex needs to reflect spatial restraints of varying accuracy and resolution that originate from vastly different experiments and theoretical considerations (Fig. 4, and Boxes 2 and 3). To this end, it is useful to express structure determination as an optimization problem. In this view, 3D models that are consistent with the input information are calculated by optimizing a scoring function. The three components of this approach are: representation of an assembly; a scoring function consisting of individual spatial restraints; and optimization of the scoring function to obtain the models. Figure 5a illustrates how the subunits of a hypothetical complex (left) can be assembled through optimization with respect to restraints from a variety of methods to obtain the final assembly model (right). Each subunit in an assembly can be represented by a set of points that depend on what is known about the subunit. If an experimentally determined structure of a protein is available or a comparative protein structure model can be calculated, each atom can be represented by its own point. If protein domains can be assigned based on biochemical characterization or bioinformatics analysis (for example, by scanning against a sequence database of domains or by prediction of transmembrane spanning domains), a single point represents each domain. Otherwise, a single point can represent the whole subunit.

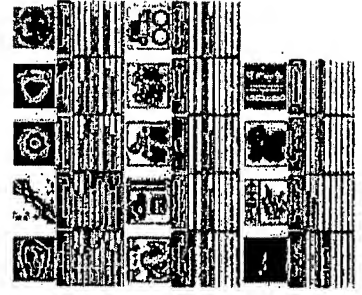


Figure 4 Experimental and theoretical methods that can provide information about a macromolecular assembly structure. [Full legend](#)

[High resolution image and legend \(136k\)](#)

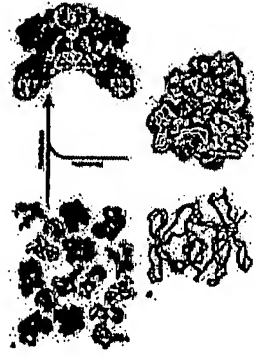


Figure 5 Hybrid approaches to structure determination of macromolecular complexes.
[Full legend](#)

[High resolution image and legend \(75k\)](#)

The most important aspect of the calculation is to accurately capture all of the existing experimental and theoretical information about the structure of a modelled assembly. For example, the shape, density and symmetry of a complex may be derived from EM; upper distance bounds on residues from different subunits may be obtained from X-ray crystallography or NMR spectroscopy and chemical crosslinking; and protein-protein contact restraints may be obtained from immuno-purification with mass spectroscopy and bioinformatics analysis of an alignment of homologous sequences. An 'ensemble' of models that minimize violations of the input restraints can be obtained by optimizing the scoring function, relying on an optimization method such as simulated annealing with molecular dynamics applied in Cartesian space. Because the optimization is likely to be stochastic, a large number of models need to be calculated and assessed. Examples of predicting assembly structures through satisfaction of varied spatial restraints include the *Escherichia coli* 30S ribosomal subunit⁵¹ and the yeast exosome⁵².

A sample study that illustrates some of the points made above is the hybrid assembly of the 80S ribosome (Fig. 5b). A partial molecular model of the whole yeast ribosome was calculated by fitting atomic ribosomal RNA and comparative protein structure models into the electron density of the 80S ribosomal particle, obtained by EM at 15-Å resolution⁵³. Most of the models for 40 out of the 75 ribosomal proteins were based on approximately 30% sequence identity to their template structures. Typical accuracy of a comparative model in this range of sequence similarity is indicated by a comparison of a model for a domain in protein L2 from *Bacillus stearothermophilus* with the actual structure. The fitting of the subunits into the electron density was made possible by the atomic structures of the whole small and large ribosomal subunits from archaea.

Visualizing complexes using electron tomography

Electron tomography is by no means a new imaging technology, but it has only recently gathered momentum^{54, 55} (Fig. 6). With the advent of computer-controlled electron microscopes and the automation of elaborate image acquisition procedures, it became possible to obtain molecular-resolution tomograms of structures as large and complex as whole prokaryotic cells or thin eukaryotic cells embedded in amorphous ice⁵⁶. Non-invasive imaging of whole, vitrified cells is where electron tomography can make a unique contribution and will probably have the greatest impact. The emerging picture of the cell is one of a giant supra-molecular assembly; but on the nanoscale, the cytoplasm is mostly an uncharted territory. Just as high-resolution 3D structures of macromolecules provide valuable insights into their working, a better understanding of cellular functions will arise from the ability to visualize macromolecules in an unperturbed cellular context.

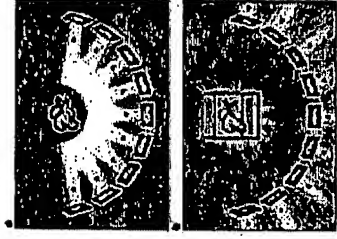


Figure 6 Principle of electron tomography. [Full legend](#)

[High resolution image and legend \(55k\)](#)

Tomograms of cells at molecular resolution are essentially 3D images of the cell's entire proteome. They reveal information about the spatial relationships of macromolecules in the cytoplasm, the 'interactome'. But exploitation of this information is confronted with two problems. Cryo-tomograms are contaminated by substantial residual noise and distorted by missing data resulting from the restricted tilt range. Moreover, the cytoplasm is very densely populated ('molecular crowding'), with molecules literally touching each other⁵⁷. Under these conditions, segmentation and feature extraction based on visual inspection is usually impossible, except for some easily recognizable features, such as membranes and the cytoskeleton.

Nevertheless, pattern-recognition techniques can be used, in one guise or another, to detect and identify specific molecules⁵⁸. Provided that a high- or medium-resolution structure of the molecule of interest is available, it can be used as a template to perform a systematic search of the reconstructed volume for matching structures (Fig. 7). Such a molecular signature-based approach, while computationally demanding, can be efficiently parallelized. Once the spatial coordinates of a complex in a cell have been determined, sub-tomograms that encompass the complex and its neighbourhood can be extracted for further analysis and averaging. Multivariate statistical analysis of such sub-tomograms can be used to explore variations in their functional environment⁵⁹.



Figure 7 Mapping the spatial distribution of complexes and their interactions within cells.

[Full legend](#)

[High resolution image and legend \(44k\)](#)

The feasibility of template matching has been demonstrated with 'phantom cells' (lipid vesicles filled with macromolecules), which provide a realistic experimental scenario and facilitate an assessment of the fidelity of the approach. With the current (non-isotropic) resolution of 4–5 nm, one can address only larger ($M_r > 400K$) complexes in a cellular context. To widen the scope of cellular tomography, it will be necessary to improve the resolution. Theoretical considerations⁶⁰ and ongoing instrumental improvements (such as liquid helium versus liquid nitrogen temperature, improved detectors and dual-axis tilting) make a resolution near 2 nm a realistic goal⁶¹.

Perspectives

The possibility seems now assured of assembling a structural picture that can be 'zoomed' continuously from the details of atomic models all the way up to the full complexity of an intact cell. Structural genomics will bring us closer to a comprehensive dictionary of proteins in the foreseeable future, while EM techniques and hybrid approaches will allow us to assemble proteins as words into meaningful sentences. A comprehensive description of large complexes will generally require the use of a number of experimental

models ([Box 2](#)), underpinned by a variety of theoretical approaches ([Box 3](#)) to maximize efficiency, completeness, accuracy and resolution of the experimental determination of assembly composition and structure. In conjunction with the non-invasive 3D imaging of whole cells, these approaches might ultimately enable us to read the molecular book of the cell.

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